PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C07K 14/62, A61K 28/28

A1

(11) International Publication Number: WO 96/29344

(43) International Publication Date: 26 September 1996 (26.09.96)

(21) International Application Number: PCT/DK96/00107

(22) International Filing Date: 18 March 1996 (18.03.96)

(30) Priority Data:
0276/95 17 March 1995 (17.03.95) DK

(71) Applicant (*for all designated States except US*): NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsværd (DK).

(72) Inventors; and
(75) Inventors/Applicants (*for US only*): MARKUSSEN, Jan [DK/DK]; Kikudbakken 7, DK-2730 Herlev (DK). JONASSEN, Ib [DK/DK]; Kirkevænget 2, DK-2500 Valby (DK). HAVELUND, Svend [DK/DK]; Kurvej 38, DK-2880 Bagsværd (DK). BRANDT, Jakob [DK/DK]; Tjørnevangen 1, st., DK-2700 Brønshøj (DK). KURTZHALS, Peter [DK/US]; Apartment A411, 20 Chapel Street, Brookline, MA 02146 (US). HANSEN, Per, Hertz [DK/DK]; Nybrovej 222, DK-2800 Lyngby (DK). KAARSHOLM, Niels, Christian [DK/DK]; Clausholmvej 38, DK-2720 Vanløse (DK).

(74) Common Representative: NOVO NORDISK A/S; Corporate Patents, Novo Allé, DK-2880 Bagsværd (DK).

(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: INSULIN DERIVATIVES

(57) Abstract

Insulin derivatives in which a lipophilic group having from 12 to 40 carbon atoms is attached to the α-amino group of the N-terminal amino acid in the B-chain or to the carboxy group of the C-terminal amino acid in the B-chain have a protracted profile of action.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgystan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic			SE	Sweden
CG	Congo	KR	Republic of Korea	SG	Singapore
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LR	Liberia	SZ	Swaziland
CS	Czechoslovakia	LT	Lithuania	TD	Chad
CZ	Czech Republic	LU	Luxembourg	TG	Togo
DE	Germany	LV	Latvia	TJ	Tajikistan
DK	Denmark	MC	Monaco	TT	Trinidad and Tobago
EE	Estonia	MD	Republic of Moldova	UA	Ukraine
ES	Spain	MG	Madagascar	UG	Uganda
FI	Finland	ML	Mali	US	United States of America
FR	France	MN	Mongolia	UZ	Uzbekistan
GA	Gabon	MR	Mauritania	VN	Viet Nam

INSULIN DERIVATIVES

FIELD OF THE INVENTION

The present invention relates to novel human insulin derivatives which are soluble and have a protracted profile of action, to a method of providing such derivatives, to pharmaceutical compositions containing them, and to the use of such insulin derivatives in the treatment of diabetes.

BACKGROUND OF THE INVENTION

Many diabetic patients are treated with multiple daily insulin injections in a regimen comprising one or two daily injections of a protracted insulin to cover the basal requirement supplemented by bolus injections of a rapid acting insulin to cover the meal- related requirements.

Protracted insulin compositions are well known in the art. Thus, one main type of protracted insulin compositions comprises injectable aqueous suspensions of insulin crystals or amorphous insulin. In these compositions, the insulin compounds utilized typically are protamine insulin, zinc insulin or protamine zinc insulin.

Certain drawbacks are associated with the use of insulin suspensions. Thus, in order to secure an accurate dosing, the insulin particles must be suspended homogeneously by gentle shaking before a defined volume of the suspension is withdrawn from a vial or expelled from a cartridge. Also, for the storage of insulin suspensions, the temperature must be kept within more narrow limits than for insulin solutions in order to avoid lump formation or coagulation.

While it was earlier believed that protamines were non-immunogenic, it has now turned out that protamines can be immunogenic in man and that their use for medical purposes may

lead to formation of antibodies (Samuel et al., Studies on the immunogenicity of protamines in humans and experimental animals by means of a micro-complement fixation test, Clin. Exp. Immunol. 33, pp. 252-260 (1978)).

Also, evidence has been found that the protamine-insulin complex is itself immunogenic (Kurtz et al., Circulating IgG antibody to protamine in patients treated with protamine-insulins. Diabetologica 25, pp. 322-324 (1983)). Therefore, with some patients the use of protracted insulin compositions containing protamines must be avoided.

Another type of protracted insulin compositions are solutions having a pH value below physiological pH from which the insulin will precipitate because of the rise in the pH value when the solution is injected. A drawback is that the solid particles of the insulin act as a local irritant causing inflammation of the tissue at the site of injection.

WO 91/12817 (Novo Nordisk A/S) discloses protracted, soluble insulin compositions comprising insulin complexes of cobalt(III). The protraction of these complexes is only intermediate and the bioavailability is reduced.

Human insulin has three primary amino groups: the N-terminal group of the A-chain and of the B-chain and the ϵ-amino group of Lys^{B29}. Several insulin derivatives which are substituted in one or more of these groups are known in the prior art. Thus, US Patent No. 3,528,960 (Eli Lilly) relates to N-carboxyaroyl insulins in which one, two or three primary amino groups of the insulin molecule has a carboxyaroyl group. No specifically $N^{\epsilon B29}$-substituted insulins are disclosed.

According to GB Patent No. 1.492.997 (Nat. Res. Dev. Corp.), it has been found that insulin with a carbamyl substitution at $N^{\epsilon B29}$ has an improved profile of hypoglycaemic effect.

JP laid-open patent application No. 1-254699 (Kodama Co., Ltd.) discloses insulin wherein a fatty acid is bound to the amino group of Phe^{B1} or to the ϵ-amino group of Lys^{B29} or to both of these. The stated purpose of the derivatisation is to obtain a pharmacologically acceptable, stable insulin preparation.

Insulins, which in the B30 position has an amino acid having at least five carbon atoms which cannot necessarily be coded for by a triplet of nucleotides, are described in JP laid-open patent application No. 57-067548 (Shionogi). The insulin analogues are claimed to be useful in the treatment of diabetes mellitus, particularly in patients who are insulin resistant due to generation of bovine or swine insulin antibodies.

US 5,359,030 (Ekwuribe, Protein Delivery, Inc.) describes conjugation-stabilized polypeptide compositions for oral or parenteral administration comprising a polypeptide covalently coupled with a polymer including a linear polyalkylene moiety and a lipophilic moiety, said moieties being arranged so relative to each other that the polypeptide has an enhanced in vivo resistance to enzymatic degradation.

EP 511600 A2 relates i.a. to protein derivatives of the formula [protein][Z]$_n$ wherein [protein] represents a protein having n amino residues each derivable from an amino group by removal of one of its hydrogen atoms, in stead of amino groups, [Z] is a residue represented by the formula -CO-W-COOH wherein W is a divalent long chain hydrocarbon group which may also contain certain hetero atoms and n represents an average of the number of amide bonds between [Z] and [protein]. It is mentioned that the protein derivatives of the invention have an extremely prolonged serum half-life as compared with the proteins from which they are derived and that they exhibit no antigenicity. It is also mentioned, that insulin is one of the proteins from which derivatives according to the invention can be made, but no specific insulin derivatives are disclosed in EP 511600 nor is there any indication of a preferred [Z] or (a) preferred

position(s) in which [Z] should be introduced in order to obtain useful insulin derivatives.

In the present specification, whenever the term insulin is used in a plural or a generic sense it is intended to encompass both naturally occurring insulins and insulin analogues and derivatives thereof. By "insulin derivative" as used herein is meant a polypeptide having a molecular structure similar to that of human insulin including the disulphide bridges between Cys^{A7} and Cys^{B7} and between Cys^{A20} and Cys^{B19} and an internal disulphide bridge between Cys^{A6} and Cys^{A11}, and which have insulin activity.

However, there still is a need for protracted injectable insulin compositions which are solutions and contain insulins which stay in solution after injection and possess minimal inflammatory and immunogenic properties.

One object of the present invention is to provide human insulin derivatives, with a protracted profile of action, which are soluble at physiological pH values.

Another object of the present invention is to provide a pharmaceutical composition comprising the human insulin derivatives according to the invention.

It is a further object of the invention to provide a method of making the human insulin derivatives of the invention.

SUMMARY OF THE INVENTION

Surprisingly, it has turned out that certain insulin derivatives, wherein either the amino group of the N-terminal amino acid of the B-chain has a lipophilic substituent comprising from 12 to 40 carbon atoms attached, or wherein the carboxylic acid group of the C-terminal amino acid of the B-chain has a lipophilic substituent comprising from 12 to 40

carbon atoms attached, have a protracted profile of action and are soluble at physiological pH values.

Accordingly, in its broadest aspect, the present invention relates to an insulin derivative having the following sequence:

```
A-Chain                           S-----------------------S
                                  |  7                    |
Gly-Ile-Val-Glu-Gln-Cys-Cys-Thr-Ser-Ile-Cys-Ser-
 1   2   3   4   5   6   |   8   9   10  11  12
                         S
                         |
B-Chain                  S
                         |
Xaa-Xaa-Xaa-Gln-His-Leu-Cys-Gly-Ser-His-Leu-Val-
 1   2   3   4   5   6   7   8   9   10  11  12

A-Chain (contd.)
                             20
Leu-Tyr-Gln-Leu-Glu-Asn-Tyr-Cys-Xaa      (SEQ ID NO:1)
 13  14  15  16  17  18  19  |   21
                         +---S
                         |
B-Chain (contd.)         S
                         |
Glu-Ala-Leu-Tyr-Leu-Val-Cys-Gly-Glu-Arg-Gly-Phe-
 13  14  15  16  17  18  19  20  21  22  23  24

B-Chain (contd.)

Phe-Xaa-Xaa-Xaa-Xaa-Xaa                  (SEQ ID NO:2)
 25  26  27  28  29  30
```

wherein

Xaa at position A21 is any codable amino acid except Lys, Arg and Cys;

Xaa at positions B1, B2, B3, B26, B27, B28 and B29 are, independent of each other, any codable amino acid except Cys or deleted;

Xaa at position B30 is any codable amino acid except Cys, a dipeptide comprising no Cys or Arg, a tripeptide comprising no Cys or Arg, a tetrapeptide comprising no Cys or Arg or deleted; and either the amino group of the N-terminal amino acid of the B-chain has a lipophilic group, W, attached to it which group has from 12 to 40 carbon atoms and optionally

contains a group which can be negatively charged or the carboxyl group of the C-terminal amino acid of the B-chain has a lipophilic group, Z, attached to it which group has from 12 to 40 carbon atoms and optionally contains a group which can be negatively charged with the proviso that if one or more of the amino acids at position B1, B2 and B3 is (are) deleted then the number of the N-terminal amino acid is found by counting down from Cys^{B7} which is always assigned the number 7 and that

(a) when B1-B2-B3 is Phe-Val-Asn and A21 is Asn and B26-B27-B28-B29-B30 is Tyr-Thr-Pro-Lys-Thr or Tyr-Thr-Pro-Lys-Ala, then said W or Z always contains a group which can be negatively charged; and

(b) when B29 and B30 are deleted and a group Z as defined above is present at the C-terminal amino acid of the B-chain and neither B1, B2 nor B3 is deleted then B1-B2 is different from Phe-Val or B26-B27-B28 is different from Tyr-Thr-Pro or both B1-B2 and B26-B27-B28 are different from said sequences; and

(c) when B29 and B30 are deleted and a group Z as defined above is present at the C-terminal amino acid of the B-chain and one of B1, B2 or B3 is deleted then the N-terminal amino acid of the B-chain is different from Val or the sequence B26-B27-B28 is different from Tyr-Thr-Pro or both the N-terminal amino acid of the B-chain and the sequence B26-B27-B28 are different from Val and Tyr-Thr-Pro respectively.

In a preferred embodiment, the present invention relates to an insulin derivative having the following sequence:

```
A-Chain                      S-----------------------S
                             |   7                   |
Gly-Ile-Val-Glu-Gln-Cys-Cys-Thr-Ser-Ile-Cys-Ser-
 1   2   3   4   5   6   |   8   9   10  11  12
                         S
                         |
B-Chain                  S
                         |
Xaa-Xaa-Xaa-Gln-His-Leu-Cys-Gly-Ser-His-Leu-Val-
 1   2   3   4   5   6   7   8   9   10  11  12
```

```
A-Chain (contd.)
                                              20
Leu-Tyr-Gln-Leu-Glu-Asn-Tyr-Cys-Xaa        (SEQ ID NO:1)
 13  14  15  16  17  18  19  |   21
                         ┌───S
                         |
B-Chain (contd.)         S
                         |
Glu-Ala-Leu-Tyr-Leu-Val-Cys-Gly-Glu-Arg-Gly-Phe-
 13  14  15  16  17  18  19  20  21  22  23  24

B-Chain (contd.)

Phe-Xaa-Xaa-Xaa-Xaa-Xaa                  (SEQ ID NO:2)
 25  26  27  28  29  30
```

wherein

Xaa at position A21 is any codable amino acid except Lys, Arg and Cys;

Xaa at positions B1, B2, B3, B26, B27, B28, B29 and B30 are, independent of each other, any codable amino acid except Cys or deleted; and either the amino group of the N-terminal amino acid of the B-chain has a lipophilic group, W, attached to it which group has from 12 to 40 carbon atoms and optionally contains a group which can be negatively charged or the carboxyl group of the C-terminal amino acid of the B-chain has a lipophilic group, Z, attached to it which group has from 12 to 40 carbon atoms and optionally contains a group which can be negatively charged with the proviso that if one or more of the amino acids at position B1, B2 and B3 is (are) deleted then the number of the N-terminal amino acid is found by counting down from Cys^{B7} which is always assigned the number 7 and that

(a) when B1-B2-B3 is Phe-Val-Asn and A21 is Asn and B26-B27-B28-B29-B30 is Tyr-Thr-Pro-Lys-Thr or Tyr-Thr-Pro-Lys-Ala, then said W or Z always contains a group which can be negatively charged; and

(b) when B29 and B30 are deleted and a group Z as defined above is present at the C-terminal amino acid of the B-chain and neither B1, B2 nor B3 is deleted then B1-B2 is different from

Phe-Val or B26-B27-B28 is different from Tyr-Thr-Pro or both B1-B2 and B26-B27-B28 are different from said sequences; and

(c) when B29 and B30 are deleted and a group Z as defined above is present at the C-terminal amino acid of the B-chain and one of B1, B2 or B3 is deleted then the N-terminal amino acid of the B-chain is different from Val or the sequence B26-B27-B28 is different from Tyr-Thr-Pro or both the N-terminal amino acid of the B-chain and the sequence B26-B27-B28 are different from Val and Tyr-Thr-Pro respectively.

When a lipophilic group, W, is attached to the α-amino group of the N-terminal amino acid of the B-chain, then the bond between the α-amino group and W is preferably an amide bond in which the N-terminal amino group of the B-chain constitutes the amine moiety and a group contained in W constitutes the carboxyl moiety.

When a lipophilic group, Z, is attached to the carboxyl group of the C-terminal amino acid of the B-chain, then the bond between the carboxyl group and Z is preferably an amide bond in which the C-terminal carboxyl group constitutes the carboxyl moiety and an amino group contained in Z constitutes the amine moiety.

In another preferred embodiment, the invention relates to an insulin derivative as described above wherein a lipophilic group, W, is attached to the α-amino group of the N-terminal amino acid of the B-chain.

In another preferred embodiment, the invention relates to a insulin derivative as described above wherein a lipophilic group, Z, is attached to the carboxyl group of the C-terminal amino acid of the B-chain.

In another preferred embodiment, the invention relates to an insulin derivative wherein the amino acid at position A21 is

selected from the group comprising Ala, Asn, Gln, Glu, Gly and Ser.

In another preferred embodiment, the invention relates to an insulin derivative wherein the amino acid at position B1 is Phe.

In another preferred embodiment, the invention relates to an insulin derivative wherein the amino acid at position B1 is deleted.

In another preferred embodiment, the invention relates to an insulin derivative wherein the amino acid at position B2 is selected from the group comprising Ala and Val.

In another preferred embodiment, the invention relates to an insulin derivative wherein the amino acid at position B3 is selected from the group comprising Asn, Gln, Glu and Thr.

In another preferred embodiment, the invention relates to an insulin derivative wherein the amino acid at position B26 is Tyr.

In another preferred embodiment, the invention relates to an insulin derivative wherein the amino acid at position B27 is Thr.

In another preferred embodiment, the invention relates to an insulin derivative wherein the amino acid at position B28 is Pro.

In another preferred embodiment, the invention relates to an insulin derivative wherein the amino acid at position B29 is Lys or Thr.

In another preferred embodiment, the invention relates to an insulin derivative wherein the amino acid at position B28 is Lys and the amino acid at position B29 is Pro.

In another preferred embodiment, the invention relates to an insulin derivative wherein the amino acid at position B30 is Thr or ε-acylated Lys.

In another preferred embodiment, the invention relates to an insulin derivative wherein Xaa at position 30 in SEQ ID NO:2 designates the dipeptide Thr-Lys.

In another preferred embodiment, the invention relates to an insulin derivative wherein Xaa at position 30 in SEQ ID NO:2 designates the dipeptide Gly-Lys.

In another preferred embodiment, the invention relates to an insulin derivative wherein Xaa at position 30 in SEQ ID NO:2 designates the tripeptide Glu-Ser-Lys.

In another preferred embodiment, the invention relates to an insulin derivative wherein Xaa at position 30 in SEQ ID NO:2 designates the tripeptide Thr-Gly-Lys.

In another preferred embodiment, the invention relates to an insulin derivative wherein Xaa at position 30 in SEQ ID NO:2 designates the tetrapeptide Thr-Gly-Gly-Lys.

In another preferred embodiment, the invention relates to an insulin derivative wherein Xaa at position 30 in SEQ ID NO:2 designates the tetrapeptide Thr-Glu-Gly-Lys.

In another preferred embodiment, the invention relates to an insulin derivative wherein Xaa at position 30 in SEQ ID NO:2 designates the tetrapeptide Gly-Asp-Thr-Lys.

In another preferred embodiment, the invention relates to an insulin derivative wherein the C-terminal amino acid of the B-chain is ε-acylated Lys and the amino acid next to the C-terminal amino acid is Gly.

In another preferred embodiment, the invention relates to an insulin derivative wherein the parent insulin is a des(B30) insulin.

In another preferred embodiment, the invention relates to an insulin derivative wherein the parent insulin is des(B30) human insulin.

In another preferred embodiment, the invention relates to an insulin derivative wherein the parent insulin is a des(B28-B30) insulin.

In another preferred embodiment, the invention relates to an insulin derivative wherein the parent insulin is a des(B27-B30) insulin.

In another preferred embodiment, the invention relates to an insulin derivative wherein the parent insulin is a des(B26-B30) insulin.

In another preferred embodiment, the invention relates to an insulin derivative wherein the amino acid at position B28 is Pro and the amino acid at position B29 is Thr.

In another preferred embodiment, the invention relates to an insulin derivative which has a group, W, as mentioned above, attached to the N-terminal α-amino group of its B-chain, W being a group of the general formula $CH_3(CH_2)_nCH(COOH)NH-CO(CH_2)_2CO-$ wherein n is an integer from 9 to 15.

In another preferred embodiment, the invention relates to an insulin derivative which has a group, W, as mentioned above, attached to the N-terminal α-amino group of its B-chain, W being a group of the general formula $CH_3(CH_2)_rCO-NHCH(COOH)(CH_2)_2CO-$ wherein r is an integer from 9 to 15.

In another preferred embodiment, the invention relates to an insulin derivative which has a group, W, as mentioned above,

attached to the N-terminal α-amino group of its B-chain, W being a group of the general formula $CH_3(CH_2)_sCO$-$NHCH((CH_2)_2COOH)CO$- wherein s is an integer from 9 to 15.

In another preferred embodiment, the invention relates to an insulin derivative which has a group, Z, as mentioned above, attached to the C-terminal amino acid of its B-chain, wherein Z is a group of the general formula $-NHCH(COOH)(CH_2)_4NH$-$CO(CH_2)_mCH_3$ wherein m is an integer from 8 to 18, that is, Z is a N^{ϵ}-acylated lysine residue.

In another preferred embodiment, the invention relates to an insulin derivative which has a group, Z, as mentioned above, attached to the C-terminal amino acid of its B-chain, wherein Z is a group of the general formula $-NHCH(COOH)(CH_2)_4NH$-$COCH((CH_2)_2COOH)NH$-$CO(CH_2)_pCH_3$ wherein p is an integer from 10 to 16.

In another preferred embodiment, the invention relates to an insulin derivative which has a group, Z, as mentioned above, attached to the C-terminal amino acid of its B-chain, wherein Z is a group of the general formula $-NHCH(COOH)(CH_2)_4NH$-$CO(CH_2)_2CH(COOH)NH$-$CO(CH_2)_qCH_3$ wherein q is an integer from 10 to 16.

In another preferred embodiment, the invention relates to an insulin derivative which has a group, Z, as mentioned above, which comprises a partly or completely hydrogenated cyclopentanophenanthrene skeleton.

In another preferred embodiment, the invention relates to an insulin derivative which has a group, Z, as mentioned above, which is an acylated amino acid, in particular acylated lysine.

In another preferred embodiment, the invention relates to Thr^{B29} human insulin with a group Z as described above attached to the C-terminal amino acid of its B-chain.

In another preferred embodiment, the invention relates to des(B28-B30) human insulin with a group Z as described above attached to the C-terminal amino acid of its B-chain.

In another preferred embodiment, the invention relates to des(B27-B30) human insulin with a group Z as described above attached to the C-terminal amino acid of its B-chain.

In another preferred embodiment, the invention relates to des(B26-B30) human insulin with a group Z as described above attached to the C-terminal amino acid of its B-chain.

In another preferred embodiment, the invention relates to the use of an insulin derivative according to the invention for the preparation of a medicament for treating diabetes.

In another preferred embodiment, the invention relates to a pharmaceutical composition for the treatment of diabetes in a patient in need of such a treatment comprising a therapeutically effective amount of an insulin derivative according to the invention together with a pharmaceutically acceptable carrier.

In another preferred embodiment, the invention relates to a pharmaceutical composition for the treatment of diabetes in a patient in need of such a treatment comprising a therapeutically effective amount of an insulin derivative according to the invention, in mixture with an insulin or an insulin analogue which has a rapid onset of action, together with a pharmaceutically acceptable carrier.

In another preferred embodiment, the invention relates to a pharmaceutical composition comprising an insulin derivative according to the invention which is soluble at physiological pH values.

In another preferred embodiment, the invention relates to a pharmaceutical composition comprising an insulin derivative

according to the invention which is soluble at pH values in the interval from about 6.5 to about 8.5.

In another preferred embodiment, the invention relates to a protracted pharmaceutical composition comprising an insulin derivative according to the invention.

In another preferred embodiment, the invention relates to a pharmaceutical composition which is a solution containing from about 120 nmol/ml to about 1200 nmol/ml, preferably about 600 nmol/ml of an insulin derivative according to the invention.

In another preferred embodiment, the invention relates to a method of treating diabetes in a patient in need of such a treatment comprising administering to the patient a therapeutically effective amount of an insulin derivative according to this invention together with a pharmaceutically acceptable carrier.

In another preferred embodiment, the invention relates to a method of treating diabetes in a patient in need of such a treatment comprising administering to the patient a therapeutically effective amount of an insulin derivative according to this invention, in mixture with an insulin or an insulin analogue which has a rapid onset of action, together with a pharmaceutically acceptable carrier.

Examples of preferred insulin derivatives according to the present invention are the following:

($N^{\epsilon B30}$-tetradecanoyl) Thr^{B29},Lys^{B30} human insulin,
($N^{\epsilon B28}$-tetradecanoyl) Lys^{B28} des(B29,B30) human insulin,
($N^{\epsilon B27}$-tetradecanoyl) Lys^{B27} des(B28-B30) human insulin and
($N^{\epsilon B26}$-tetradecanoyl) Lys^{B26} des(B27-B30) human insulin.
($N^{\epsilon B32}$-tetradecanoyl) Glu^{B30},Ser^{B31},Lys^{B32} human insulin.
($N^{\epsilon B29}$-acetyl,$N^{\epsilon B32}$-tetradecanoyl) Glu^{B30},Ser^{B31},Lys^{B32} human insulin.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention is further illustrated with reference to the appended drawing wherein

Fig. 1 shows the construction of the plasmids pKV153, pKV159, pJB173, pJB174 and pJB175;

Fig. 2a which is continued in Fig. 2b shows the sequence of pMT742, position 907 to 1500, and the oligonucleotides #94, #593, #2371 and #3075 used for PCR1A, PCR1B and PCR1C of Example 1. The 138 amino acid sequence corresponding to the MF alpha prepro-leader (amino acids Nos. 1-85) and an insulin precursor which has the amino acid sequence B(1-29)AlaAlaLysA(1-21) wherein A(1-21) is the A chain of human insulin and B(1-29) is the B chain of human insulin in which Thr(B30) is missing, is shown below the coding sequence (amino acids Nos. 86-138).

DETAILED DESCRIPTION OF THE INVENTION

Terminology

The three letter codes and one letter codes for the amino acid residues used herein are those stated in J. Biol. Chem. <u>243</u>, p. 3558 (1968).

In the DNA sequences, A is adenine, C is cytosine, G is guanine, and T is thymine.

The following acronyms are used:

DMSO for dimethyl sulphoxide,
DMF for dimethylformamide,
Boc for *tert*-butoxycarbonyl,
NMP for 1-methyl-2-pyrrolidone,
TFA for trifluoroacetic acid,
X-OSu for an N-hydroxysuccinimid ester,
X for an acyl group,

RP-HPLC for reversed phase high performance liquid chromatography.

Preparation of lipophilic insulin derivatives

The insulin derivatives according to the present invention can be prepared i.a. as described in the following:

1. Insulin derivatives featuring in position B30 an amino acid residue which can be coded for by the genetic code, e.g. threonine (human insulin) or alanine (porcine insulin).

1.1 Insulins modified by attachment of a lipophilic group, W, to the N-terminal amino group, starting from human insulin.

Human insulin is treated with a Boc-reagent (e.g. di-*tert*-butyl dicarbonate) to form (A1,B29)-diBoc human insulin, i.e., human insulin in which the N-terminal end of the A-chain and the ϵ-amino group of Lys^{B29} are protected by a Boc-group. After an optional purification, e.g. by HPLC, an acyl group is introduced in the α-amino group of Phe^{B1} by allowing the product to react with a N-hydroxysuccinimide ester of the formula W-OSu wherein W is an acyl group as defined in the above to be introduced at the N-terminal α-amino group of the B-chain. In the final step, TFA is used to remove the Boc-groups and the product, $N^{\alpha B1}$-W human insulin, is isolated.

2. Insulin derivatives with no amino acid residue in position B30, i.e. des(B30) insulins.

2.1 Starting from human insulin or porcine insulin.

On treatment with carboxypeptidase A in ammonium buffer, human insulin and porcine insulin both yield des(B30) insulin. After an optional purification, the des(B30) insulin is treated with a Boc-reagent (e.g. di-*tert*-butyl dicarbonate) to form

(A1,B29)-diBoc des(B30) insulin. After an optional purification, e.g. by HPLC, an acyl group is introduced in the α-amino group of the amino acid in position B1 by allowing the product to react with a N-hydroxysuccinimide ester of the formula W-OSu wherein W is the acyl group to be introduced. In the final step, TFA is used to remove the Boc-groups and the product, ($N^{\alpha B1}$-W) des(B30) insulin, is isolated.

2.2 Starting from a single chain human insulin precursor.

A single chain human insulin precursor, which is extended in position B1 with an extension (Ext) which is connected to B1 via an arginine residue and which has a bridge from a C-terminal lysine in position B26, B27, B28 or B30 to A1 can be a used as starting material. Preferably, the bridge is a peptide of the formula Y_n-Arg, where Y is a codable amino acid except cysteine, lysine and arginine, and n is zero or an integer between 1 and 35. When n>1, the Y's may designate different amino acids. Preferred examples of the bridge from Lys in position B26, B27, B28 or B30 to A1 are: AlaAlaArg, SerArg, SerAspAspAlaArg and Arg (European Patent No. 163529). Treatment of such a precursor of the general formula Ext-Arg-B(1-Q)-Y_n-Arg-A(1-21), wherein Q is 26, 27, 28 or 30, with a lysyl endopeptidase, e.g. *Achromobacter lyticus* protease, yields Ext-Arg-B(1-Q) Y_n-Arg-A(1-21) insulin. Acylation of this intermediate with a N-hydroxysuccinimide ester of the general formula X-OSu wherein X is an acyl group, introduces the acyl group X in the ϵ-amino group of Lys^{BQ}, and in the N-terminal amino group of the A-chain and the B-chain to give ($N^{\epsilon BQ}$-X) X-Ext-Arg-B(1-Q) X-Y_n-Arg-A(1-21) insulin. This intermediate on treatment with trypsin in mixture of water and a suitable organic solvent, e.g. DMF, DMSO or a lower alcohol, gives the desired derivative, Z-human insulin wherein Z is $Lys^{\epsilon BQ}$-X.

Pharmaceutical compositions

Pharmaceutical compositions containing a human insulin derivative according to the present invention may be administered parenterally to patients in need of such a treatment. Parenteral administration may be performed by subcutaneous, intramuscular or intravenous injection by means of a syringe, optionally a pen-like syringe. Alternatively, parenteral administration can be performed by means of an infusion pump. A further option is a composition which may be a powder or a liquid for the administration of the human insulin derivative in the form of a nasal spray.

Pharmaceutical compositions containing a compound of the present invention may be prepared by conventional techniques, e.g. as described in Remington's Pharmaceutical Sciences, 1985.

Thus, the injectable human insulin compositions of the invention can be prepared using the conventional techniques of the pharmaceutical industry which involves dissolving and mixing the ingredients as appropriate to give the desired end product.

Thus, according to one procedure, the human insulin derivative is dissolved in an amount of water which is somewhat less than the final volume of the composition to be prepared. An isotonic agent, a preservative and a buffer is added as required and the pH value of the solution is adjusted - if necessary - using an acid, e.g. hydrochloric acid, or a base, e.g. aqueous sodium hydroxide as needed. Finally, the volume of the solution is adjusted with water to give the desired concentration of the ingredients.

Examples of isotonic agents are sodium chloride, mannitol and glycerol.

Examples of preservatives are phenol, m-cresol, methyl p-hydroxybenzoate and benzyl alcohol.

Examples of suitable buffers are sodium acetate and sodium phosphate.

Preferred pharmaceutical compositions of the particular insulins of the present invention are solutions hexameric complexes. Typically the hexameric complexes are stabilized by two or more zinc ions and three or more molecules of a phenolic compound like phenol or meta-cresol or mixtures thereof per hexamer.

In a particular embodiment, a composition is provided which contains two different insulins, one having a protracted profile of action and one having a rapid onset of action, in the form of soluble hexameric complexes. Typically the hexameric complexes are stabilized by two or more zinc ions and three or more molecules of a phenolic compound like phenol or meta-cresol or mixtures thereof per hexamer. The complexes are mixtures of hexamers of the particular insulins and mixed hexamers in which the ratio between the two different insulins is from 1:5 to 5:1.

A composition for nasal administration of an insulin derivative according to the present invention may, for example, be prepared as described in European Patent No. 272097 (to Novo Nordisk A/S).

The insulin compositions of this invention can be used in the treatment of diabetes. The optimal dose level for any patient will depend on a variety of factors including the efficacy of the specific human insulin derivative employed, the age, body weight, physical activity, and diet of the patient, on a possible combination with other drugs, and on the severity of the case of diabetes. It is recommended that the daily dosage of the human insulin derivative of this invention be determined for each individual patient by those skilled in the art in a similar way as for known insulin compositions.

Where expedient, the human insulin derivatives of this invention may be used in mixture with other types of insulin, e.g. human insulin or porcine insulin or insulin analogues with a more rapid onset of action. Examples of such insulin analogues are described e.g. in the European patent applications having the publication Nos. EP 214826 (Novo Nordisk A/S), EP 375437 (Novo Nordisk A/S) and EP 383472 (Eli Lilly & Co.).

The present invention is further illustrated by the following examples which, however, are not to be construed as limiting the scope of protection. The features disclosed in the foregoing description and in the following examples may, both separately and in any combination thereof, be material for realizing the invention in diverse forms thereof.

EXAMPLES

Plasmids and DNA material

All expression plasmids are of the cPOT type. Such plasmids are described in EP patent application No. 171 142 and are characterised in containing the *Schizosaccharomyces pombe* triose phosphate isomerase gene (POT) for the purpose of plasmid selection and stabilisation. A plasmid containing the POT-gene is available from a deposited E. coli strain (ATCC 39685). The plasmids furthermore contain the *S. cerevisiae* triose phosphate isomerase promoter and terminator (P_{TPI} and T_{TPI}). They are identical to pMT742 (Egel-Mitani, M et al., Gene 73 (1988) 113-120) (see Fig. 1) except for the region defined by the EcoRI-XbaI restriction sites encompassing the coding region for MF alpha prepro leader/product. The EcoRI/XbaI fragment of pMT742 itself encodes the Mating Factor (MF) alpha prepro-leader sequence of *Saccharomyces cerevisiae* followed by the insulin precursor MI3 which has a Ala-Ala-Lys bridge connecting B29 and A1 (i.e. B(1-29)-Ala-Ala-Lys-A(1-21))(see Fig. 2)

Synthetic DNA fragments were synthesised on an automatic DNA synthesizer (Applied Biosystems model 380A) using phosphoramidite chemistry and commercially available reagents (Beaucage, S.L. and Caruthers, M.H., Tetrahedron Letters 22 (1981) 1859-1869).

All other methods and materials used common state of the art knowledge (see, e.g. Sambrook, J., Fritsch, E.F. and Maniatis, T. Molecular Cloning: A Laboratory Manual, Cold Spring Harbour Laboratory Press, New York, 1989).

Analytical

Molecular masses of insulin precursors prepared were obtained by mass spectroscopy (MS), either by plasma desorption mass spectrometry (PDMS) using Bio-Ion 20 instrument (Bio-Ion Nordic AB, Uppsala, Sweden) or electrospray mass spectrometry (ESMS) using an API III Biomolecular Mass Analyzer (Perkin Elmer Sciex Instruments, Thornhill, Canada).

The lipophilicity of an insulin derivative relative to human insulin, k'_{rel}, was measured on a LiChrosorb® RP18 (5μm, 250x4 mm) HPLC column by isocratic elution at 40°C using mixtures of A) 0.1 M sodium phosphate buffer, pH 7.3, containing 10% acetonitrile, and B) 50% acetonitrile in water. The elution was monitored by the absorption of the eluate at 214 nm. Void time, t_0, was found by injecting 0.1 mM sodium nitrate. Retention time for human insulin, t_{human}, was adjusted to at least $2t_0$ by varying the ratio between the A and B solutions. k'_{rel} is defined as $(t_{derivative}-t_0)/(t_{human}-t_0)$.

As a measure of the protraction of the compounds of the invention, the disappearance rate in pigs was studied and $T_{50\%}$ was determined. $T_{50\%}$ is the time when 50% of the A14 Tyr(^{125}I)-labeled analogue has disappeared from the site of injection as measured with an external γ-counter (Ribel, U et al., The Pig as a Model for Subcutaneous Absorption in Man. In: M. serrano-Rios and P.J. Lefebre (Eds): Diabetes 1985; Proceedings of the

12th Congress of the International Diabetes Federation, Madrid, Spain, 1985 (Excerpta Medica, Amsterdam, (1986) 891-96).

EXAMPLE 1

Synthesis of Glu-Glu-Ala-Glu-Ala-Glu-Ala-Glu-Pro-Lys-Ala-Thr-Arg-B(1-29)-Ser-Asp-Asp-Ala-Arg-A(1-21) insulin precursor from yeast strain yKV153 using the *S.cerevisiae* MF alpha prepro-leader.

The following oligonucleotides were synthesised:

#593 5'-CCAAGTACAAAGCTTCAACCAAGTGGGAACCGCACAAGTGTTGGTTAA
CGAATCTTGTAGCCTTTGGTTCAGCTTCAGCTTCAGCTTCTTCTCTTTTAT
CCAAAGAAACACC-3'

#94 5'-TAAATCTATAACTACAAAAAACACATA-3'

#3075 5'-TTGGTTGAAGCTTTGTACTTGGTTTGCGGTGAAAGAGGTTTCTTCTAC
ACTCCTAAGTCTGACGATGCTAGAGGTATTG-3'

#2371 5'-TTAATCTTAGTTTCTAGAGCCTGCGGG-3'

The following two Polymerase Chain Reactions (PCR) were performed using Gene Amp PCR reagent kit (Perkin Elmer, 761 Main Avewalk, CT, USA) according to the manufacturer's instructions (see Fig. 2).

<u>PCR1A:</u>

0.2 μl of pMT742 plasmid template
4.0 μl of oligonucleotide #593 (100 pmol)
4.0 μl of oligonucleotide #94 (100 pmol)

10.0 μl of 10x PCR buffer
10.0 μl of 2.5 Mm dNTP
0.5 μl of Taq polymerase enzyme
71.3 μl of water

PCR1B:

0.2 μl of pMT742 plasmid template
4.0 μl of oligonucleotide #3075 (100 pmol)
4.0 μl of oligonucleotide #2371 (100 pmol)
10.0 μl of 10x PCR buffer
10.0 μl of 2.5 mM dNTP
0.5 μl of Taq polymerase enzyme
71.3 μl of water

In both cases two cycles were performed at 94 °C for 1 min., 45 °C for 1 min. and 72 °C for 1 min. and subsequently followed by 11 cycles: 94 °C for 1 min., 55 °C for 1 min., 72 °C for 1 min.

20 μl of each PCR mixture was loaded onto a 2% agarose gel and subjected to electrophoresis using standard techniques (Sambrook et al., Molecular cloning, Cold Spring Harbour Laboratory Press, 1989). Resulting DNA fragments (452 bp from PCR1A and 170 bp from PCR1B) were cut out of the agarose gel and isolated using the Gene Clean kit (Bio101 Inc., PO BOX 2284, La Jolla, CA, USA) according to the manufacturer's instructions. The purified DNA fragments were dissolved in 100 μl of water.

The following PCR was performed

PCR1C:

1.0 μl of DNA fragment from PCR1A
1.0 μl of DNA fragment from PCR1B
10.0 μl of 10 x PCR buffer
10.0 μl of 2.5 mM dNTP

0.5 μl of Taq polymerase enzyme
69.5 μl of water

Two cycles were performed at 94 °C for 1 min., 45 °C for 1 min. and 72 °C for 1 min.

Subsequently 4 μl of oligonucleotide #94 (100 pmol) and 4.0 μl of oligonucleotide #2371 (100 pmol) was added and 15 cycles were performed at 94 °C for 1 min., 55 °C for 1 min. and 72 °C for 1 min.

The PCR mixture was loaded onto a 1% agarose gel and the resulting 594 bp fragment was purified using the Gene Clean kit as described. The purified PCR DNA fragment was dissolved in 20 μl of water and restriction endonuclease buffer and cut with the restriction endonucleases *Eco*RI and *Xba*I (New England Biolabs, Inc. MA, USA). The resulting 550 bp *Eco*RI/*Xba*I restriction fragment was run on agarose gel and isolated and purified using the Gene clean kit.

In two separate restriction endonuclease digestions the plasmid pMT742 was cut with i) restriction endonucleases *Apa*I and *Xba*I and ii) with restriction endonucleases *Apa*I and *Eco*RI. From these digestions the 8.6 kb *Apa*I/*Xba*I restriction fragment and the 2.1 kb *Apa*I/*Eco*RI restriction fragment was isolated.

The three fragments (i.e. the 550 bp *Eco*RI/*Xba*I restriction fragment from PCR1C and the 8.6 kb *Apa*I/*Xba*I restriction fragment and 2.1 kb *Apa*I/*Eco*RI restriction fragment from pMT742) were ligated together using T4 DNA ligase and standard conditions (Sambrook et al., Molecular cloning, Cold Spring Harbour Laboratory Press, 1989)(see Fig. 1). The ligation mixture was transformed into competent *E. coli* cells (Ap^{r-}) followed by selection for ampicillin resistance. Plasmids were isolated from the resulting *E. coli* colonies using standard DNA miniprep technique (Sambrook et al., Molecular cloning, Cold Spring Harbour Laboratory Press, 1989) and checked with appropriate restriction endonucleases (*i.e. Eco*RI, *Apa*I and

*Xba*I). The selected plasmid designated pKV153 was shown by DNA sequencing analysis (using the Sequenase kit from U.S. Biochemical Corp. according to the manufacturer's recommendations) to contain the correct sequence encoding the MF alpha prepro-leader/Glu-Glu-Ala-Glu-Ala-Glu-Ala-Glu-Pro-Lys-Ala-Thr-Arg-B(1-29)-Ser-Asp-Asp-Ala-Arg-A(1-21) insulin precursor.

pKV153 was transformed into S. cerevisiae strain MT663 and selected for growth on glucose as described in European patent application having the publication No. 214826.

The resulting yeast strain was designated yKV153 and was verified to produce Glu-Glu-Ala-Glu-Ala-Glu-Ala-Glu-Pro-Lys-Ala-Thr-Arg-B(1-29)-Ser-Asp-Asp-Ala-Arg-A(1-21) insulin precursor in the culture media by HPLC and mass spectroscopy.

EXAMPLE 2

Synthesis of $Lys^{B30}(N^{\epsilon}$-tetradecanoyl) Thr^{B29} human insulin.

2a. Synthesis of Glu-Glu-Ala-Glu-Ala-Glu-Ala-Glu-Pro-Lys-Ala-Thr-Arg-B(1-28)-Thr-Lys-Ser-Asp-Asp-Ala-Arg-A(1-21) insulin precursor from yeast strain yKV159 using the *S.cerevisiae* MF alpha prepro-leader.

The following oligonucleotides were synthesised:

#3881 5'-TTGGTTGAAGCTTTGTACTTGGTTTGCGGTGAAAGAGGTTTCTTCTAC ACTCCTACCAAGTCTGACGATGCTAGAGGTATTGTCG-3'

#2371 5'-TTAATCTTAGTTTCTAGAGCCTGCGGG-3'

The following Polymerase Chain Reaction (PCR) was performed using Gene Amp PCR reagent kit as described above.

<u>PCR2:</u>

0.2 µl of pKV153 plasmid template (from Example 1)
4.0 µl of oligonucleotide #3881 (100 pmol)
4.0 µl of oligonucleotide #2371 (100 pmol)
10.0 µl of 10x PCR buffer
10.0 µl of 2.5 mM dNTP
0.5 µl of Taq polymerase enzyme
71.3 µl of water

Two cycles were performed at 94 °C for 1 min., 45 °C for 1 min. and 72 °C for 1 min. and subsequently followed by 11 cycles at 94 °C for 1 min., 55 °C for 1 min., 72 °C for 1 min.

The PCR mixture was loaded onto a 2% agarose gel and the resulting 174 bp fragment was purified using the Gene Clean kit as described. The purified PCR DNA fragment was dissolved in 20 µl of water and restriction endonuclease buffer and cut with the restriction endonucleases *Hin*dIII and *Xba*I. The resulting 153 bp *Hin*dIII/*Xba*I restriction fragment was run on agarose gel and isolated and purified using the Gene Clean kit.

In two separate restriction endonuclease digestions pMT742 was cut with restriction endonucleases *Apa*I and *Xba*I whereas pKV153 (from Example 1) was cut with restriction endonucleases *Apa*I and *Eco*RI. From this digestions the 8.6 kb *Apa*I/*Xba*I restriction fragment from pMT742 and the 2.1 kb *Apa*I/*Eco*RI restriction fragment from pKV153 was isolated.

The three fragments (i.e. the 550 bp *Eco*RI/*Xba*I restriction fragment from PCR2 and the 8.6 kb *Apa*I/*Xba*I restriction fragment from pMT748 and the 2.1 kb *Apa*I/*Eco*RI restriction fragment from pKV153) were ligated together using T4 DNA ligase as described above (see Fig. 1). The ligation mixture was transformed into competent *E. coli* cells (Ap^{r-}) followed by selection for ampicillin resistance. Plasmids were isolated from the resulting *E. coli* colonies and checked for appropriate restriction endonucleases (*i.e.* *Hin*dIII, *Apa*I and *Xba*I)as described.

The selected plasmid designated pKV159 was shown by DNA sequencing analysis to contain the correct sequence encoding the MF alpha prepro-leader/Glu-Glu-Ala-Glu-Ala-Glu-Ala-Glu-Pro-Lys-Ala-Thr-Arg-B(1-28)-Thr-Lys-Ser-Asp-Asp-Ala-Arg-A(1-21) insulin precursor.

pKV159 was transformed into S. cerevisiae strain MT663 and selected for growth on glucose as described.

The resulting yeast strain was designated yKV159 and was verified to produce Glu-Glu-Ala-Glu-Ala-Glu-Ala-Glu-Pro-Lys-Ala-Thr-Arg-B(1-28)-Thr-Lys-Ser-Asp-Asp-Ala-Arg-A(1-21) insulin precursor in the culture media by HPLC and mass spectroscopy.

2b. Isolation of Glu-Glu-Ala-Glu-Ala-Glu-Ala-Glu-Pro-Lys-Ala-Thr-Arg-B(1-28)-Thr-Lys-Ser-Asp-Asp-Ala-Arg-A(1-21) insulin precursor.

The yKV159 strain was fermented for 72 hours and five litres of broth were collected. Yeast cells were removed by centrifugation, the pH value was adjusted to 3.0 using sulphuric acid and the insulin precursor was concentrated on a Pharmacia Strealine® 50 column packed with 300 ml of Streamline® SP ion exchanger. After wash with 25 mM citrate buffer, pH value 3.6, the precursor was eluted by 0.5 M NH_3 and the fraction from 300 to 600 ml was collected. The pH value was adjusted to 2.5 and the precursor was purified by RP-HPLC using 15 μ spherical C18 silica of 100 Å pore size and 0.2 M Na_2SO_4, 0.04 M H_3PO_4 as buffer, and using a gradient from 23 to 33% acetonitrile. The precursor eluted at about 27-28% acetonitrile. The pool containing the central major peak of the precursor was desalted by gel filtration on Sephadex® G-50 F in 0.5 M acetic acid, and the precursor isolated by lyophilization. Yield: 486 mg.

2c. Synthesis of Ala-Thr-Arg-B(1-28)-Thr-Lys, Ser-Asp-Asp-Ala-Arg-A(1-21) insulin.

The 486 mg of single-chain precursor obtained as described above were dissolved in 30 ml of 0.05 M glutamate buffer at pH 9.0, and 3 ml of immobilized A. lyticus protease gel were added (see PCT/DK94/00347, page 45). After gentle stirring for 5 hours at 30°C, the gel was removed by filtration and the double-chain, extended insulin was crystallized by the addition of 10 ml of ethanol, 845 mg of trisodium citrate dihydrate and 78 mg of zinc chloride. After adjustment of the pH value to 6.1 and storage at 4°C overnight the crystals were collected by centrifugation, washed twice with isopropanol and dried *in vacuo*. Yield: 450 mg.

2d. Synthesis of $N^{\alpha A-5}$,$N^{\alpha B-3}$,$N^{\epsilon B30}$-tris(tetradecanoyl) Ala-Thr-Arg-B(1-28)-Thr-Lys, Ser-Asp-Asp-Ala-Arg-A(1-21) insulin.

450 mg of the double-chain, extended insulin, obtained as described above, were dissolved in a mixture of 3.15 ml of DMSO and 0.69 ml of 2 M diisopropylethylamine in NMP. The solution was cooled to 15°C and 0.69 ml of 0.3 M tetradecanoic acid N-hydroxysuccinimide ester in DMSO/NMP (1:1, v/v) was added. After 2 hours at 15°C, the reaction was stopped by addition of 112 ml of 0.01 M glycine buffer in ethanol/water (60:40, v/v) and the pH value adjusted to 10.0. The triacylated intermediate was not isolated.

2e. Synthesis of Lys^{B30}(N^{ϵ}-tetradecanoyl) Thr^{B29} human insulin.

To the solution from the previous step was added 5 ml of immobilized trypsin gel (see PCT/DK94/00347, page 46). After gentle stirring at 15°C for 16 hours, the gel was removed by filtration, the pH value was adjusted to 9.0 and the solution was applied to a 2.5 x 25 cm column of QAE-Sephadex® A-25. Isocratic elution was performed at a rate of 17.3 ml/h using a 0.12 M NH_4Cl buffer in ethanol/water (60:40, v/v) adjusted to pH 9.0 with NH_3. The title compound emerged from the column after 650 ml, and a pool from 650 to 754 ml was collected. Finally, the buffer was changed to 0.01 M NH_4HCO_3 by gel

filtration on Sephadex® G-50 Fine, and the product isolated in the dry state by lyophilization. Yield: 91 mg.

Molecular mass of the title compound, found by MS: 6020 ± 6, theory: 6018.
Molecular mass of the B-chain, found by MS: 3642 ± 5, theory: 3640.
Molecular mass of the C-terminal fragment of B-chain digested by V8 protease, found by MS: 1326 ± 2, theory: 1326.
Relative lipophilicity, k'_{rel} = 113.
Disappearance half-life, $T_{50\%}$, after subcutaneous injection in pigs: 20.3 ± 5.2 hours (n=6).

EXAMPLE 3

Synthesis of Lys^{B28}(N^{ϵ}-tetradecanoyl) des(B29-B30) human insulin.

3a. Synthesis of Glu-Glu-Ala-Glu-Ala-Glu-Ala-Glu-Pro-Lys-Ala-Thr-Arg-B(1-27)-Lys-Ser-Asp-Asp-Ala-Arg-A(1-21) insulin precursor from yeast strain yJB173 using the *S. cerevisiae* MF alpha prepro-leader.

The following oligonucleotides were synthesised:

#627 5'-CACTTGGTTGAAGCTTTGTACTTGGTTTGCGGTGAAAGAGGTTTCTTC
TACACTAAGTCTGACGATGCTAG-3'

#2371 5'-TTAATCTTAGTTTCTAGAGCCTGCGGG-3'

The DNA encoding Glu-Glu-Ala-Glu-Ala-Glu-Ala-Glu-Pro-Lys-Ala-Thr-Arg-B(1-27)-Lys-Ser-Asp-Asp-Ala-Arg-A(1-21) was constructed in the same manner as described in Example 2 by substituting oligonucleotide #3881 with oligonucleotide #627.

The resulting plasmid was designated pJB173 and the yeast strain expressing Glu-Glu-Ala-Glu-Ala-Glu-Ala-Glu-Pro-Lys-Ala-

Thr-Arg-B(1-27)-Lys-Ser-Asp-Asp-Ala-Arg-A(1-21) was designated yJB173.

3b. Isolation of Glu-Glu-Ala-Glu-Ala-Glu-Ala-Glu-Pro-Lys-Ala-Thr-Arg-B(1-27)-Lys-Ser-Asp-Asp-Ala-Arg-A(1-21) insulin precursor.

The yJB173 strain was fermented for 72 hours and 4.8 litres of broth were collected. Yeast cells were removed by centrifugation and the pH value was adjusted to 3.0 using sulphuric acid. The conductivity was 7.8 mS/cm. The insulin precursor was concentrated using a Pharmacia Streamline® 50 column packed with 300 ml of Streamline® SP ion exchanger. After wash with 25 mM citrate buffer, pH value 3.6, the precursor was eluted by 0.5 M NH_3 and the fraction from 300 to 600 ml was collected. Free ammonia was evaporated *in vacuo* at room temperature and the pH value of the resulting 280 ml of solution was adjusted to 9.0 with hydrochloric acid.

3c. Synthesis of Ala-Thr-Arg-B(1-27)-Lys, Ser-Asp-Asp-Ala-Arg-A(1-21) insulin.

To the 280 ml of solution containing 118 mg of the single-chain precursor, obtained as described above, were added 3 ml of immobilized A. lyticus protease gel (see PCT/DK94/00347, page 45). After gentle stirring for 24 hours at 30°C the gel was removed by filtration. The pH value was adjusted to 3.5 and the solution was filtered though a Milipore® 0.45 μ filter. The double-chain, extended insulin was purified in 2 runs by RP-HPLC using a 2x20 cm column packed with 15 μ spherical C18 silica of 100 Å pore size and 0.2 M Na_2SO_4, 0.04 M H_3PO_4, pH 3.5 as buffer, and using a gradient from 23 to 33% acetonitrile at a rate of 4 ml/min and a column temperature of 40°C. The double-chain, extended insulin eluted at about 30-31% acetonitrile. The acetonitrile was removed from the combined pools of 70 ml by evaporation *in vacuo*, and salts were removed by gelfiltration using a 5x47 cm column of Sephadex G-25 in 0.5

M acetic acid. The double-chain, extended insulin was isolated by lyophilization. Yield: 110 mg.

3d. Synthesis of $N^{\alpha A-5}$,$N^{\alpha B-3}$,$N^{\epsilon B28}$-tris(tetradecanoyl) Ala-Thr-Arg-B(1-27)-Lys, Ser-Asp-Asp-Ala-Arg-A(1-21) insulin.

110 mg of the double-chain, extended insulin obtained as described above were dissolved in a mixture of 0.84 ml of DMSO and 0.275 ml of 2 M diisopropylethylamine in NMP. The solution was cooled to 15°C and 0.185 ml of 0.3 M tetradecanoic acid N-hydroxysuccinimide ester in DMSO/NMP (1:1, v/v) was added. After 2 hour at 15°C, the reaction was stopped by addition of 32.5 ml of 0.01 M glycine buffer in ethanol/water (60:40, v/v) and the pH value adjusted to 10.0. The triacylated intermediate was not isolated.

3e. Synthesis of Lys^{B28}(N^{ϵ}-tetradecanoyl) des(B29-B30) human insulin.

To the resulting solution from the previous step was added 1.5 ml of immobilized trypsin gel (see PCT/DK94/00347, page 46). After gentle stirring at 15°C for 18 hours, the gel was removed by filtration, the pH value adjusted to 9.0 and the solution applied to a 1.5 x 21 cm column of QAE-Sephadex® A-25. Isocratic elution was performed at a rate of 10 ml/h using a 0.12 M NH_4Cl buffer in ethanol/water (60:40, v/v) adjusted to pH 9.0 with NH_3. The title compound emerged from the column after 250-390 ml, peaking at 330 ml. Finally, the buffer was changed to 0.01 M NH_4HCO_3 by gel filtration using Sephadex® G-50 Fine, and the product was isolated in the dry state by lyophilization. Yield: 47 mg.

Molecular mass of the title compound, found by MS: 5820 ± 2, theory: 5819.

Molecular mass of the B-chain, found by MS: 3444 ± 4, theory: 3442.

Molecular mass of the C-terminal fragment of B-chain digested by V8 protease, found by MS: 1128 ± 2, theory: 1128.

Relative lipophilicity, k'_{rel} = 121.
Disappearance half-life, $T_{50\%}$, after subcutaneous injection in pigs: 19.6 ± 3.6 h (n=4).

EXAMPLE 4

Synthesis of $Lys^{B27}(N^{\epsilon}$ -tetradecanoyl) des(B28-B30) human insulin.

4a. Synthesis of Glu-Glu-Ala-Glu-Ala-Glu-Ala-Glu-Pro-Lys-Ala-Thr-Arg-B(1-26)-Lys-Ser-Asp-Asp-Ala-Arg-A(1-21) insulin precursor from yeast strain yJB174 using the *S. cerevisiae* MF alpha prepro-leader.

The following oligonucleotides were synthesised:

#628 5-'CACTTGGTTGAAGCTTTGTACTTGGTTTGCGGTGAAAGAGGTTTCTTC TACAAGTCTGACGATGCTAG-3'

#2371 5'-TTAATCTTAGTTTCTAGAGCCTGCGGG-3'

The DNA encoding Glu-Glu-Ala-Glu-Ala-Glu-Ala-Glu-Pro-Lys-Ala-Thr-Arg-B(1-26)-Lys-Ser-Asp-Asp-Ala-Arg-A(1-21) was constructed in the same manner as described in Example 2 by substituting oligonucleotide #3881 with oligonucleotide #628.

The resulting plasmid was designated pJB174 and the yeast strain expressing Glu-Glu-Ala-Glu-Ala-Glu-Ala-Glu-Pro-Lys-Ala-Thr-Arg-B(1-26)-Lys-Ser-Asp-Asp-Ala-Arg-A(1-21) was designated yJB174.

4b. Isolation of Glu-Glu-Ala-Glu-Ala-Glu-Ala-Glu-Pro-Lys-Ala-Thr-Arg-B(1-26)-Lys-Ser-Asp-Asp-Ala-Arg-A(1-21) insulin precursor.

The yJB174 strain was fermented for 72 hours and 3.5 litres of broth were collected. Yeast cells were removed by

centrifugation, the pH value was adjusted to 3.0 using sulphuric acid and the solution was diluted with water to 8 litres in order to decrease the salt concentration. The resulting conductivity was 7.9 mS/cm. The insulin precursor was concentrated using a Pharmacia Streamline® 50 column packed with 300 ml of Streamline® SP ion exchanger. After wash with 25 mM citrate buffer, pH 3.6, the precursor was eluted by 0.5 M NH_3 and the fraction from 300 to 600 ml was collected. Free ammonia was evaporated *in vacuo* at room temperature and the pH value of the resulting 280 ml was adjusted to 9.0 with hydrochloric acid.

4c. Synthesis of Ala-Thr-Arg-B(1-26)-Lys, Ser-Asp-Asp-Ala-Arg-A(1-21) insulin.

To the 280 ml of solution of the single-chain precursor obtained as described above was added 3 ml of immobilized A. lyticus protease gel (see PCT/DK94/00347, page 45). After gentle stirring for 13 hours at 30°C the gel was removed by filtration. The pH value was adjusted to 2.5 and the solution was filtered though a Milipore® 0.45 μ filter. The double-chain, extended insulin was purified in 4 runs by RP-HPLC using a 2x20 cm column packed with 15 μ spherical C18 silica of 100 Å pore size and 0.2 M Na_2SO_4, 0.04 M H_3PO_4, pH 2.5 as buffer, and using a gradient from 24 to 33% acetonitrile. The double-chain, extended insulin eluted at about 30-31% acetonitrile. The acetonitrile was removed from the combined pools by evaporation *in vacuo*, and the salts were removed by gelfiltration using a 5x47 cm column of Sephadex G-25 in 0.5 M acetic acid. The double-chain, extended insulin was isolated by lyophilization. Yield: 69 mg.

4d. Synthesis of $N^{\alpha A-5}$,$N^{\alpha B-3}$,$N^{\epsilon B27}$-tris(tetradecanoyl) Ala-Thr-Arg-B(1-26)-Lys, Ser-Asp-Asp-Ala-Arg-A(1-21) insulin.

62 mg of the double-chain, extended insulin obtained as described under 4e was dissolved in a mixture of 0.44 ml of DMSO and 0.15 ml of 2 M diisopropylethylamine in NMP. The

solution was cooled to 15°C and 0.096 ml of 0.3 M tetradecanoic acid N-hydroxysuccinimide ester in DMSO/NMP (1:1, v/v) was added. After 2 hours at 15°C the reaction was stopped by addition of 17 ml of 0.01 M glycine buffer in ethanol/water (60:40, v/v) and the pH value adjusted to 10.0. The triacylated intermediate was not isolated.

4e. Synthesis of Lys^{B27}(N^{ϵ} -tetradecanoyl) des(B28-B30) human insulin.

To the solution from the previous step was added 1 ml of immobilized trypsin gel (see PCT/DK94/00347, page 46). After gentle stirring at 15°C for 26 hours, the gel was removed by filtration, the pH value adjusted to 9.0 and the solution applied to a 1.5 x 25.5 cm column of QAE-Sephadex® A-25. Isocratic elution was performed at a rate of 17.3 ml/h using a 0.12 M NH_4Cl buffer in ethanol/water (60:40, v/v) adjusted to pH 9.0 with NH_3. The title compound emerged from the column after 360 ml, and a pool from 272 to 455 ml was collected. Finally, the buffer was changed to 0.01 M NH_4HCO_3 by gel filtration on Sephadex® G-50 Fine, and the product isolated in the dry state by lyophilization. Yield: 38 mg.

Molecular mass of the title compound, found by MS: 5720 ± 6, theory: 5718.
Molecular mass of the B-chain, found by MS: 3342 ± 4, theory: 3340.
Molecular mass of the C-terminal fragment of B-chain digested by V8 protease, found by MS: 1027 ± 2, theory: 1027.
Relative lipophilicity, k'_{rel} = 151.
Disappearance half-life, $T_{50\%}$, after subcutaneous injection in pigs: 15.2 ± 2.2 h (n=5).

EXAMPLE 5

Synthesis of Lys^{B26}(N^{ϵ} -tetradecanoyl) des(B27-B30) human insulin.

5a. Synthesis of Glu-Glu-Ala-Glu-Ala-Glu-Ala-Glu-Pro-Lys-Ala-Thr-Arg-B(1-25)-Lys-Ser-Asp-Asp-Ala-Arg-A(1-21) insulin precursor from yeast strain yJB175 using the *S. cerevisiae* MF alpha prepro-leader.

The following oligonucleotides were synthesised:

#629 5'-CACTTGGTTGAAGCTTTGTACTTGGTTTGCGGTGAAAGAGGTTTCTTC AAAGTCTGACGATGCTAG-3'

#2371 5'-TTAATCTTAGTTTCTAGAGCCTGCGGG-3'

The DNA encoding Glu-Glu-Ala-Glu-Ala-Glu-Ala-Glu-Pro-Lys-Ala-Thr-Arg-B(1-25)-Lys-Ser-Asp-Asp-Ala-Arg-A(1-21) was constructed in the same manner as described in Example 2 by substituting oligonucleotide #3881 with oligonucleotide #629.

The resulting plasmid was designated pJB175 and the yeast strain expressing Glu-Glu-Ala-Glu-Ala-Glu-Ala-Glu-Pro-Lys-Ala-Thr-Arg-B(1-25)-Lys-Ser-Asp-Asp-Ala-Arg-A(1-21) was designated yJB175.

5b. Isolation of Glu-Glu-Ala-Glu-Ala-Glu-Ala-Glu-Pro-Lys-Ala-Thr-Arg-B(1-25)-Lys-Ser-Asp-Asp-Ala-Arg-A(1-21) insulin precursor.

The yJB175 strain was fermented for 72 hours and 3.7 litres of broth were collected. Yeast cells were removed by centrifugation, the pH value was adjusted to 3.0 using sulphuric acid and the solution was diluted with water to 8.5 litres in order to decrease the salt concentration. The resulting conductivity was 7.7 mS/cm. The insulin precursor was concentrated using a Pharmacia Strealine® 50 column packed with 300 ml of Streamline® SP ion exchanger. After wash with 25 mM citrate buffer, pH 3.6, the precursor was eluted by 0.5 M ammonia and the fraction from 300 to 600 ml was collected. Free ammonia was evaporated *in vacuo* at room temperature and the pH

value of the resulting 270 ml of solution was adjusted to 9.0 with hydrochloric acid.

5c. Synthesis of Ala-Thr-Arg-B(1-25)-Lys, Ser-Asp-Asp-Ala-Arg-A(1-21) insulin.

To the 270 ml solution of the single-chain precursor obtained as described above was added 3 ml of immobilized A. lyticus protease gel (see PCT/DK94/00347, page 45). After gentle stirring for 23 hours at 30°C, the gel was removed by filtration. The pH value was adjusted to 2.5 and the solution was filtered though a Milipore® 0.45 μ filter. The double-chain, extended insulin was purified in 4 runs by RP-HPLC using a 2x20 cm column packed with 15 μ spherical C18 silica of 100 Å pore size and 0.2 M Na_2SO_4, 0.04 M H_3PO_4, pH 3.5 as buffer, and using a gradient from 24 to 33% acetonitrile. The double-chain, extended insulin eluted at about 29-31% acetonitrile. The acetonitrile was removed from the combined pools by evaporation *in vacuo*, and the salts were removed by gelfiltration using a 5x47 cm column of Sephadex® G-25 in 0.5 M acetic acid. The double-chain, extended insulin was isolated by lyophilization. Yield: 81 mg.

5d. Synthesis of $N^{\alpha A-5}$,$N^{\alpha B-3}$,$N^{\epsilon B26}$-tris(tetradecanoyl) Ala-Thr-Arg-B(1-25)-Lys, Ser-Asp-Asp-Ala-Arg-A(1-21) insulin.

80 mg of the double-chain, extended insulin was dissolved in a mixture of 0.56 ml of DMSO and 0.19 ml of 2 M diisopropylethylamine in NMP. The solution was cooled to 15°C and 0.124 ml of 0.3 M tetradecanoic acid N-hydroxysuccinimide ester in DMSO/NMP (1:1, v/v) was added. After 2 hour at 15°C the reaction was stopped by addition of 21.8 ml of 0.01 M glycine buffer in ethanol/water (60:40, v/v) and the pH value adjusted to 10.0. The triacylated intermediate was not isolated.

5e. Synthesis of Lys^{B26}(N^{ϵ}-tetradecanoyl) des(B27-B30), human insulin.

To the solution from the previous step was added 1 ml of immobilized trypsin gel (see PCT/DK94/00347, page 46). After gentle stirring at 15°C for 23 hours the gel was removed by filtration, the pH value was adjusted to 9.0 and the solution applied to a 1.5 x 25.5 cm column of QAE-Sephadex® A-25. Isocratic elution was performed at a rate of 19.3 ml/h using a 0.12 M NH_4Cl buffer in ethanol/water (60:40, v/v) adjusted to pH 9.0 with ammonia. The title compound emerged from the column after 320 ml, and a fraction from 320 to 535 ml was collected. Finally, the buffer was changed to 0.01 M NH_4HCO_3 by gel filtration on Sephadex® G-50 Fine, and the product isolated in the dry state by lyophilization. Yield: 25 mg.

Molecular mass of the title compound found by MS: 5555 ± 6, theory: 5555.
Molecular mass of the B-chain found by MS: 3179 ± 4, theory: 3178.
Molecular mass of the C-terminal fragment of B-chain digested by V8 protease found by MS: 864 ± 1, theory: 863.5.
Relative lipophilicity, k'_{rel} = 151.
Disappearance half-life, $T_{50\%}$, after subcutaneous injection in pigs: 14.4 ± 1.5 h (n = 5).

EXAMPLE 6

Synthesis of (N(1-carboxytridecyl)-2-amidosuccinyl)-Phe$^{\alpha B1}$ des(B30) human insulin.

A1,B29-diBoc-des(B30) human insulin (200 mg, 0.033 mmol) was dissolved in DMF (15 ml) and triethylamine (20 µl) was added. N(1-carbomethoxytridecyl)-2-amidosuccinic acid N-hydroxysuccinimide ester (16 mg, 0.033 mmol) was added and after 4 hours at room temperature the reaction mixture was evaporated *in vacuo* to dryness. The Boc groups were removed by treatment for 30 min at room temperature with trifluoroacetic acid (5 ml). The trifluoroacetic acid was removed by evaporation *in vacuo*. The residue was dissolved at 0°C in 0.1

N NaOH (20 ml). The saponification of the methyl ester was accomplished after 1 hour at 0°C. The pH value of the reaction mixture was adjusted to 5.0 by acetic acid and ethanol (5 ml) was added. The precipitate formed was isolated by centrifugation.

The title compound was purified from the precipitate by ion exchange chromatography using a 2.5 x 27 cm column of QAE-Sephadex® A25.

The precipitate was dissolved ethanol/water (60:40, v/v) (25 ml) by adjustment of the pH value to 9.8 using ammonia and the solution was applied to the column. Elution was carried out in NH_3/NH_4Cl buffers at pH 9.0, using a linear gradient from 0.12 to 0.18 M NH_4Cl in ethanol/water (60:40, v/v) and a total of 1000 ml of eluent. The UV absorbance of the eluate was monitored at 280 nm and fractions of 10 ml were collected. The title compound emerged from the column in fractions 62 to 72. The title compound was precipitated by diluting the pool with 2 volumes of water and adjusting the pH value to 5.0. After centrifugation the precipitate was washed with water and after a second centrifugation the product was dried *in vacuo*. Yield: 10 mg.

Molecular weight, found by PDMS: 6032, theory: 6032.

Relative lipophilicity, k'_{rel} = 140.

Disappearance half-life, $T_{50\%}$, after subcutaneous injection in pigs: 8.65±1.65 hours (n = 5).

EXAMPLE 7

Phe$^{\alpha B1}$-tetradecanoyl-glutamyl-glycyl des(B30) human insulin.

A1,B29-diBoc des(B30) human insulin (200 mg, 0.033 mmol) was dissolved in DMF (15 ml) and triethylamine (100 μl) was added. Myristoyl-Glu(γ-OtBu)-Gly N-hydroxysuccinimide ester (95 mg, 0.17 mmol) was added and after 4 hours at room temperature the reaction mixture was evaporated to dryness *in vacuo*. The Boc and tBu groups were removed by treatment for 30 min at room

temperature with trifluoroacetic acid (5 ml). The trifluoroacetic acid was removed by evaporation *in vacuo*.
The title compound was purified from the precipitate by RP-HPLC using a C18 silica column and eluting with a linear gradient from 16 to 64% acetonitrile in a 50 mM Tris/phosphate buffer containing 75 mM $(NH_4)_2SO_4$ at pH 7. The title compound emerged from the column at about 50% acetonitrile. The acetonitrile was evaporated *in vacuo*, and ethanol was added to 20% (v/v). Adjustment of the pH value to 5.0 caused the product to precipitate. After centrifugation the precipitate was dissolved in 10 mM NH_4HCO_3, desalted by gel filtration using Sephadex G-25 and lyophilized. Yield: 97 mg.
Molecular mass, found by PDMS: 6105, theory: 6104.

EXAMPLE 8

Synthesis of Gly^{B28},Thr^{B29},Lys^{B30}(N^{ϵ}-tetradecanoyl) human insulin.

8a. Synthesis of Glu-Glu-Ala-Glu-Ala-Glu-Ala-Glu-Pro-Lys-Ala-Thr-Arg-B(1-27)-Gly-Thr-Lys-Ser-Asp-Asp-Ala-Arg-A(1-21) insulin precursor from yeast strain yKV195 using the *S. cerevisiae* MF alpha prepro-leader.

The following oligonucleotides were synthesised:

#4790 5'-TTGGTTGAAGCTTTGTACTTGGTTTGCGGTGAAAGAGGTTTCTTCTAC
ACTGGTACCAAGTCTGACGATGCTAGAGGTATTGTCG-3'

#2371 5'-TTAATCTTAGTTTCTAGAGCCTGCGGG-3'

The DNA encoding Glu-Glu-Ala-Glu-Ala-Glu-Ala-Glu-Pro-Lys-Ala-Thr-Arg-B(1-27)-Gly-Thr-Lys-Ser-Asp-Asp-Ala-Arg-A(1-21) was constructed in the same manner as described in Example 2 by substituting oligonucleotide #3881 with oligonucleotide #4790.

The resulting plasmid was designated pKV195 and the yeast strain expressing Glu-Glu-Ala-Glu-Ala-Glu-Ala-Glu-Pro-Lys-Ala-Thr-Arg-B(1-27)-Gly-Thr-Lys-Ser-Asp-Asp-Ala-Arg-A(1-21) was designated yKV195.

8b. Isolation of Glu-Glu-Ala-Glu-Ala-Glu-Ala-Glu-Pro-Lys-Ala-Thr-Arg-B(1-27)-Gly-Thr-Lys-Ser-Asp-Asp-Ala-Arg-A(1-21) insulin precursor.

The yKV195 strain was fermented for 72 hours and 4.4 litres of broth were collected. Yeast cells were removed by centrifugation, the pH value was adjusted to 3.0 using sulphuric acid and 3.6 litres of water were added to dilute salts to a conductivity of 7.7 mS/cm. The insulin precursor was concentrated using a Pharmacia Strealine® 50 column packed with 300 ml of Streamline® SP ion exchanger. After wash with 3 litres of 25 mM citrate buffer, pH 3.6, the precursor was eluted using 0.5 M ammonia and the fraction from 300 to 600 ml was collected. Free ammonia was evaporated *in vacuo* at room temperature and the pH value of the resulting 280 ml was adjusted to 9.0 with hydrochloric acid.

8c. Synthesis of Ala-Thr-Arg-B(1-27)-Gly-Thr-Lys, Ser-Asp-Asp-Ala-Arg-A(1-21) insulin.

To the 280 ml of solution containing 300 mg of the single-chain precursor were added 3 ml of immobilized A. lyticus protease gel (see PCT/DK94/00347, page 45). After gentle stirring for 17 hours at 30°C the gel was removed by filtration. The pH value was adjusted to 3.5 and the solution was filtered though a Milipore® 0.45 μ filter. The double-chain, extended insulin was purified in 3 runs by RP-HPLC using a 2x20 cm column packed with 10 μ spherical C18 silica of 120 Å pore size and 0.2 M Na_2SO_4, 0.04 M H_3PO_4, pH 3.5 as buffer, and using a gradient from 23 to 33% acetonitrile at a rate of 4 ml/min and a column temperature of 40°C. The double-chain, extended insulin eluted at about 30-31% acetonitrile. The acetonitrile was removed from the combined pools of 70 ml by evaporation *in vacuo*, and the

salt were removed by gelfiltration using a 5x47 cm column of Sephadex® G-25 and 0.5 M acetic acid. The double-chain, extended insulin was isolated by lyophilization. Yield: 176 mg.

8d. Synthesis of $N^{\alpha A-5}$,$N^{\alpha B-3}$,$N^{\epsilon B30}$-tris(tetradecanoyl) Ala-Thr-Arg-B(1-27)-Gly-Thr-Lys, Ser-Asp-Asp-Ala-Arg-A(1-21) insulin.

176 mg of the double-chain, extended insulin was dissolved in a mixture of 1.4 ml of DMSO and 0.275 ml of 2 M diisopropylethylamine in NMP. The solution was cooled to 15°C and 0.963 ml of 0.3 M tetradecanoic acid N-hydroxysuccinimide ester in DMSO/NMP (1:1, v/v) was added. After 20 hour at 15°C the reaction was stopped by addition of 50 ml of 0.01 M glycine buffer in ethanol/water (60:40, v/v) and the pH value adjusted to 10.0. The triacylated intermediate was not isolated.

8e. Synthesis of Gly^{B28},Thr^{B29},Lys^{B30}(N^{ϵ}-tetradecanoyl) human insulin.

To the solution from the previous step was added 2.5 ml of immobilized trypsin gel (see PCT/DK94/00347, page 46). After gentle stirring at 15°C for 5 hours, the gel was removed by filtration, the pH value adjusted to 9.0 and the solution applied to a 1.5 x 26.5 cm column of QAE-Sephadex® A-25. Isocratic elution was performed at a rate of 9.3 ml/h using a 0.12 M NH_4Cl buffer in ethanol/water (60:40, v/v) adjusted to pH 9.0 with ammonia. The title compound emerged from the column after 325-455 ml, peaking at 380 ml. Finally, the buffer was changed to 0.01 M NH_4HCO_3 by gel filtration using Sephadex® G-50 Fine, and the product isolated in the dry state by lyophilization. Yield: 50 mg.

Molecular mass of the title compound, found by MS: 5979 ± 6, theory: 5977.
Molecular mass of the B-chain, found by MS: 3600 ± 4, theory: 3600.
Molecular mass of the C-terminal fragment of B-chain digested by V8 protease, found by MS: 1286 ± 2, theory: 1286.

Relative lipophilicity, $k'_{rel} = 103$.
Disappearance half-life, $T_{50\%}$, after subcutaneous injection in pigs: 17 ± 2 h ($n = 4$).

EXAMPLE 9.

Synthesis of Gly^{B28},Lys^{B29}(N^{ϵ}-tetradecanoyl) human insulin.

9a. Synthesis of Glu-Glu-Ala-Glu-Ala-Glu-Ala-Glu-Pro-Lys-Ala-Thr-Arg-B(1-27)-Gly-Lys-Ser-Asp-Asp-Ala-Arg-A(1-21) insulin precursor from yeast strain yKV196 using the *S. cerevisiae* MF alpha prepro-leader.

The following oligonucleotides were synthesised:

#4791 5'-TTGGTTGAAGCTTTGTACTTGGTTTGCGGTGAAAGAGGTTTCTTCTAC
ACCGGTAAGTCTGACGATGCTAGAGGTATTGTCG-3'

#2371 5'-TTAATCTTAGTTTCTAGAGCCTGCGGG-3'

The DNA encoding Glu-Glu-Ala-Glu-Ala-Glu-Ala-Glu-Pro-Lys-Ala-Thr-Arg-B(1-27)-Gly-Lys-Ser-Asp-Asp-Ala-Arg-A(1-21) was constructed in the same manner as described in Example 2 by substituting oligonucleotide #3881 with oligonucleotide #4791.

The resulting plasmid was designated pKV196 and the yeast strain expressing Glu-Glu-Ala-Glu-Ala-Glu-Ala-Glu-Pro-Lys-Ala-Thr-Arg-B(1-27)-Gly-Lys-Ser-Asp-Asp-Ala-Arg-A(1-21) was designated yKV196.

9b. Isolation of Glu-Glu-Ala-Glu-Ala-Glu-Ala-Glu-Pro-Lys-Ala-Thr-Arg-B(1-27)-Gly-Lys-Ser-Asp-Asp-Ala-Arg-A(1-21) insulin precursor.

The yKV196 strain was fermented for 72 hours and 3.6 litres of broth were collected. Yeast cells were removed by centrifugation, the pH value was adjusted to 3.0 using

sulphuric acid and 3.4 litres of water were added to dilute salts to a conductivity of 7.7 mS/cm. The insulin precursor was concentrated to 300 ml using the procedure described in Example 8b.

9c. Synthesis of Ala-Thr-Arg-B(1-27)-Gly-Lys, Ser-Asp-Asp-Ala-Arg-A(1-21) insulin.

To the 300 ml solution at pH 9.0 containing 390 mg of the single-chain precursor were added 5 ml of immobilized A. lyticus protease gel (see PCT/DK94/00347, page 45). After gentle stirring for 40 hours at 30°C, the gel was removed by filtration. The pH value was adjusted to 3.5 and the solution was filtered though a Milipore® 0.45 μ filter. The double-chain, extended insulin was purified in 3 runs by RP-HPLC using a 2x20 cm column packed with 10 μ spherical C18 silica of 120 Å pore size and 0.2 M Na_2SO_4, 0.04 M H_3PO_4, pH 3.5 as buffer, and using a gradient from 23 to 33% acetonitrile at a rate of 4 ml/min and a column temperature of 40°C. The double-chain, extended insulin eluted at about 29% acetonitrile. The acetonitrile was removed from the combined pools of 60 ml by evaporation *in vacuo*, and the salt were removed by gelfiltration using a 5x47 cm column of Sephadex® G-25 and 0.5 M acetic acid. The double-chain, extended insulin was isolated by lyophilization. Yield: 154 mg.

9d. Synthesis of $N^{\alpha A-5}$,$N^{\alpha B-3}$,$N^{\epsilon B29}$-tris(tetradecanoyl) Ala-Thr-Arg-B(1-27)-Gly-Lys, Ser-Asp-Asp-Ala-Arg-A(1-21) insulin.

154 mg of the double-chain, extended insulin was dissolved in a mixture of 1.05 ml of DMSO and 0.329 ml of 2 M diisopropylethylamine in NMP. The solution was cooled to 15°C and 0.22 ml of 0.3 M tetradecanoic acid N-hydroxysuccinimide ester in DMSO/NMP (1:1, v/v) was added. After 2 hour at 15°C, the reaction was stopped by addition of 40 ml of 0.01 M glycine buffer in ethanol/water (60:40, v/v) and the pH value adjusted to 10.0. The triacylated intermediate was not isolated.

9e. Synthesis of Gly^{B28},Lys^{B29}(N^{ϵ}-tetradecanoyl) human insulin.

To the solution from the previous step was added 1.5 ml of immobilized trypsin gel (see PCT/DK94/00347, page 46). After gentle stirring at 15°C for 21 hours, the gel was removed by filtration, the pH value adjusted to 9.0 and the product in 43 ml solution was applied to a 1.5 x 26.0 cm column of QAE-Sephadex® A-25. Isocratic elution was performed at a rate of 9.5 ml/h using a 0.12 M NH_4Cl buffer in ethanol/water (60:40, v/v) adjusted to pH 9.0 with ammonia. The title compound emerged from the column after 190-247 ml, peaking at 237 ml. Finally, the buffer was changed to 0.01 M NH_4HCO_3 by gel filtration using Sephadex® G-50 Fine, and the product isolated in the dry state by lyophilization. Yield: 67 mg.

Molecular mass of the title compound, found by MS: 5877 ± 2, theory: 5876.
Molecular mass of the B-chain, found by MS: 3499 ± 3, theory: 3499.
Molecular mass of the C-terminal fragment of B-chain digested by V8 protease, found by MS: 1184 ± 2, theory: 1185.
Relative lipophilicity, k'_{rel} = 118.5.
Disappearance half-life, $T_{50\%}$, after subcutaneous injection in pigs: 25 ± 9 h (n = 4).

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Novo Nordisk A/S
(B) STREET: Novo Allé
(C) CITY: DK-2880 Bagsvaerd
(E) COUNTRY: Denmark
(G) TELEPHONE: +45 44448888
(H) TELEFAX: +45 44490555
(I) TELEX: 37173

(ii) TITLE OF INVENTION: ACYLATED INSULIN

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Novo Nordisk A/S
Corporate Patents
(B) STREET: Novo Alle
(C) CITY: DK-2880 Bagsvaerd
(E) COUNTRY: Denmark

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBERS: DK 0276/95
(B) FILING DATES: 17-MAR-1995

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Jørgensen, Dan et al.
(C) REFERENCE/DOCKET NUMBER: 4341-WO,DJ

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: +45 44448888
(B) TELEFAX: +45 44493256

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu
 1               5                  10                  15

Glu Asn Tyr Cys Xaa
            20
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Xaa Xaa Xaa Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr
 1               5                  10                  15

Leu Val Cys Gly Glu Arg Gly Phe Phe Xaa Xaa Xaa Xaa Xaa
            20                  25                  30
```

CLAIMS

1. An insulin derivative having the following sequence:

```
A-Chain                          S----------------------S
                                 |  7                   |
Gly-Ile-Val-Glu-Gln-Cys-Cys-Thr-Ser-Ile-Cys-Ser-
 1   2   3   4   5   6   |   8   9   10  11  12
                         S
                         |
B-Chain                  S
                         |
Xaa-Xaa-Xaa-Gln-His-Leu-Cys-Gly-Ser-His-Leu-Val-
 1   2   3   4   5   6   7   8   9   10  11  12

A-Chain (contd.)
                                20
Leu-Tyr-Gln-Leu-Glu-Asn-Tyr-Cys-Xaa      (SEQ ID NO:1)
 13  14  15  16  17  18  19  |   21
                         ----S
                         |
B-Chain (contd.)         S
                         |
Glu-Ala-Leu-Tyr-Leu-Val-Cys-Gly-Glu-Arg-Gly-Phe-
 13  14  15  16  17  18  19  20  21  22  23  24

B-Chain (contd.)

Phe-Xaa-Xaa-Xaa-Xaa-Xaa                  (SEQ ID NO:2)
 25  26  27  28  29  30
```

wherein

Xaa at position A21 is any codable amino acid except Lys, Arg and Cys;

Xaa at positions B1, B2, B3, B26, B27, B28 and B29 are, independent of each other, any codable amino acid except Cys or deleted;

Xaa at position B30 is any codable amino acid except Cys, a dipeptide comprising no Cys or Arg, a tripeptide comprising no Cys or Arg, a tetrapeptide comprising no Cys or Arg or deleted; and either the amino group of the N-terminal amino acid of the B-chain has a lipophilic group, W, attached to it which group has from 12 to 40 carbon atoms and optionally contains a group which can be negatively charged or the carboxyl group of the C-terminal amino acid of the B-chain has

a lipophilic group, Z, attached to it which group has from 12 to 40 carbon atoms and optionally contains a group which can be negatively charged with the proviso that if one or more of the amino acids at position B1, B2 and B3 is (are) deleted then the number of the N-terminal amino acid is found by counting down from Cys^{B7} which is always assigned the number 7 and that

(a) when B1-B2-B3 is Phe-Val-Asn and A21 is Asn and B26-B27-B28-B29-B30 is Tyr-Thr-Pro-Lys-Thr or Tyr-Thr-Pro-Lys-Ala, then said W or Z always contains a group which can be negatively charged; and

(b) when B29 and B30 are deleted and a group Z as defined above is present at the C-terminal amino acid of the B-chain and neither B1, B2 nor B3 is deleted then B1-B2 is different from Phe-Val or B26-B27-B28 is different from Tyr-Thr-Pro or both B1-B2 and B26-B27-B28 are different from said sequences; and

(c) when B29 and B30 are deleted and a group Z as defined above is present at the C-terminal amino acid of the B-chain and one of B1, B2 or B3 is deleted then the N-terminal amino acid of the B-chain is different from Val or the sequence B26-B27-B28 is different from Tyr-Thr-Pro or both the N-terminal amino acid of the B-chain and the sequence B26-B27-B28 are different from Val and Tyr-Thr-Pro respectively.

2. The insulin derivative according to claim 1, wherein a lipophilic group, W, is attached to the amino group of the N-terminal amino acid in the B-chain.

3. The insulin derivative according to claim 1, wherein a lipophilic group, Z, is attached to the carboxyl group of the C-terminal amino acid in the B-chain.

4. The insulin derivative according to any one of the preceding claims, wherein Xaa at position A21 designates an amino acid selected from the group comprising Ala, Asn, Gln, Glu, Gly and Ser.

5. The insulin derivative according to any one of the claims 1 to 3, wherein Xaa at position B1 designates Phe or is deleted.

6. The insulin derivative according to any one of the claims 1 to 3, wherein Xaa at position B2 designates Ala or Val.

7. The insulin derivative according to any one of the claims 1 to 3, wherein Xaa at position B3 designates an amino acid selected from the group comprising Asn, Gln, Glu, and Thr.

8. The insulin derivative according to any one of the claims 1 to 3, wherein Xaa at position B26 designates Tyr.

9. The insulin derivative according to any one of the claims 1 to 3, wherein Xaa at position B27 designates Thr.

10. The insulin derivative according to any one of the claims 1 to 3, wherein Xaa at position B28 designates Pro.

11. The insulin derivative according to any one of the claims 1 to 3, wherein Xaa at position B29 designates Lys or Thr.

12. The insulin derivative according to any one of the claims 1 to 3, wherein Xaa at position B30 designates Thr or ε-acylated Lys.

13. The insulin derivative according to any one of the claims 1 to 3, wherein Xaa at position B30 is deleted.

14. The insulin derivative according to any one of the claims 1 to 3, wherein Xaa at position B28 designates Lys and Xaa at position B29 designates Pro.

15. The insulin derivative according to any one of the claims 1 to 3, wherein Xaa at position B28 designates Pro and Xaa at position B29 designates Thr.

16. The insulin derivative according to claim 2 wherein W is attached to the amino group of the N-terminal amino acid via an amide bond.

17. The insulin derivative according to claim 16 wherein W is $CH_3(CH_2)_nCH(COOH)NH\text{-}CO(CH_2)_2CO\text{-}$ and n is an integer from 9 to 15.

18. The insulin derivative according to claim 3 wherein Z is attached to the carboxyl group of the C-terminal amino acid via an amide bond.

19. The insulin derivative according to claim 18 wherein Z is $\text{-}NHCH(COOH)(CH_2)_4NH\text{-}CO(CH_2)_mCH_3$ and m is an integer from 8 to 18.

20. The insulin derivative according to claim 19 wherein the parent insulin to which Z is attached is Thr^{B29} human insulin.

21. The insulin derivative according to claim 19 wherein the parent insulin to which Z is attached is des(B28-B30) human insulin.

22. The insulin derivative according to claim 19 wherein the parent insulin to which Z is attached is des(B27-B30) human insulin.

23. The insulin derivative according to claim 19 wherein the parent insulin to which Z is attached is des(B26-B30) human insulin.

24. An insulin derivative according to claim 1 wherein the C-terminal amino acid of the B-chain is ε-acylated Lys and the amino acid next to the C-terminal amino acid is Gly.

25. A pharmaceutical composition for the treatment of diabetes in a patient in need of such treatment, comprising a therapeutically effective amount of an insulin derivative according to claim 1 together with a pharmaceutically acceptable carrier.

26. A pharmaceutical composition for the treatment of diabetes in a patient in need of such treatment, comprising a therapeutically effective amount of an insulin derivative according to claim 1, in mixture with an insulin or an insulin analogue which has a rapid onset of action, together with a pharmaceutically acceptable carrier.

27. A method of treating diabetes in a patient in need of such a treatment, comprising administering to the patient a therapeutically effective amount of an insulin derivative according to claim 1 together with a pharmaceutically acceptable carrier.

28. A method of treating diabetes in a patient in need of such a treatment, comprising administering to the patient a therapeutically effective amount of an insulin derivative according to claim 1 in mixture with an insulin or an insulin analogue which has a rapid onset of action, together with a pharmaceutically acceptable carrier.

1/3

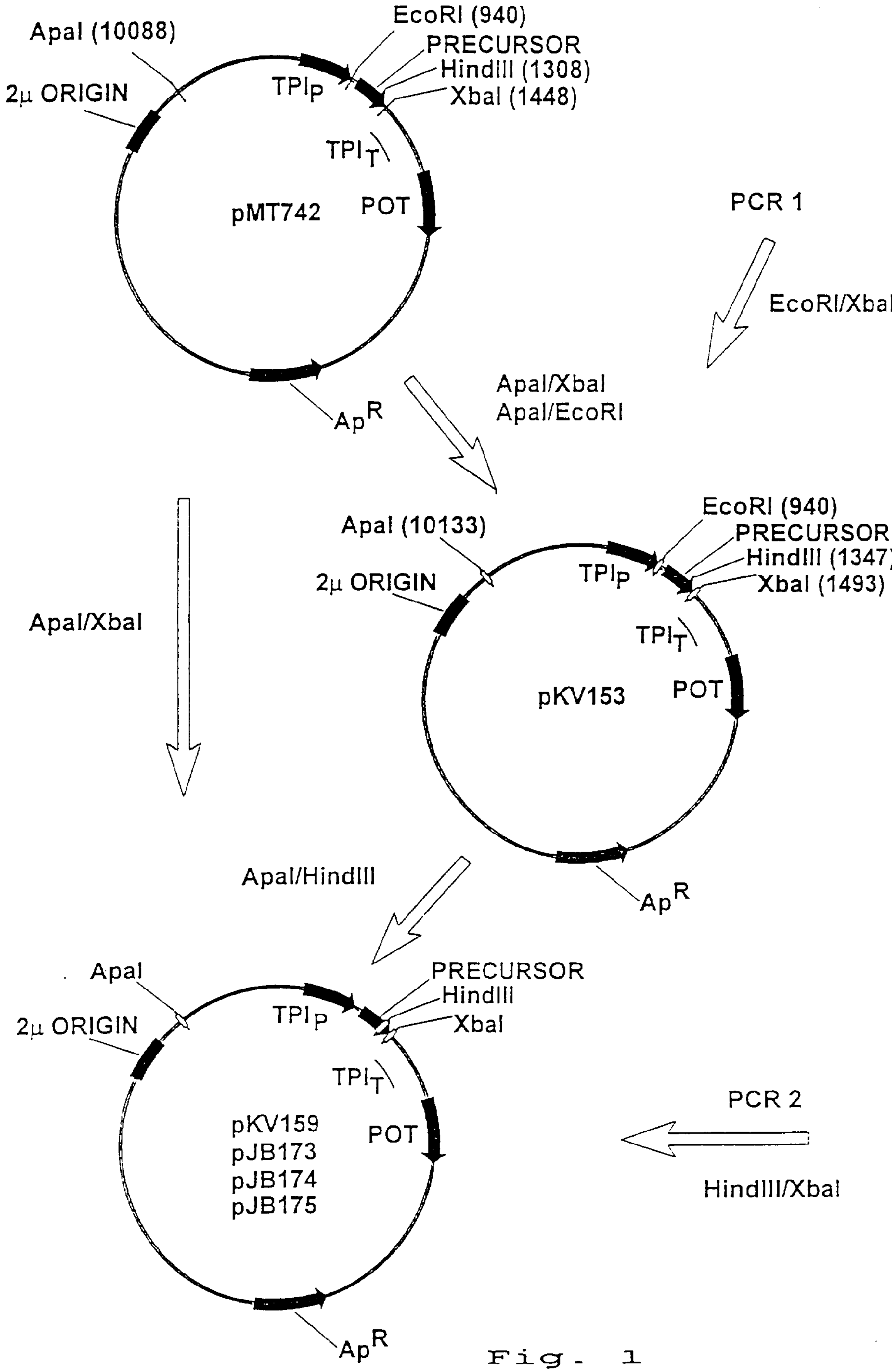
ApaI (10088)
EcoRI (940)
PRECURSOR
HindIII (1308)
XbaI (1448)
2μ ORIGIN
TPIP
TPIT
pMT742
POT
ApR
PCR 1
EcoRI/XbaI
ApaI/XbaI
ApaI/EcoRI
ApaI (10133)
EcoRI (940)
PRECURSOR
HindIII (1347)
XbaI (1493)
2μ ORIGIN
TPIP
ApaI/XbaI
TPIT
pKV153
POT
ApR
ApaI/HindIII
ApaI
PRECURSOR
HindIII
XbaI
2μ ORIGIN
TPIP
TPIT
pKV159
pJB173
pJB174
pJB175
POT
PCR 2
HindIII/XbaI
ApR

Fig. 1

2/3

```
            #94 ↓
      5'-TAAATCTATAACTACAAAAAACACATA-3'EcoRI
     5'-CTTAAATCTATAACTACAAAAAACACATACAGGAATTCCATTCAAGAATAGTTCAAACAA
 907 ---+---------+---------+---------+---------+---------+------ 966
     3'-GAATTTAGATATTGATGTTTTTTGTGTATGTCCTTAAGGTAAGTTCTTATCAAGTTTGTT

       GAAGATTACAAACTATCAATTTCATACACAATATAAACGATTAAAAGAATGAGATTTCCT
 967   ---+---------+---------+---------+---------+---------+------ 1026
       CTTCTAATGTTTGATAGTTAAAGTATGTGTTATATTTGCTAATTTTCTTACTCTAAAGGA
                                                       MetArgPhePro    4

       TCTATTTTTACTGCTGTTTTATTCGCTGCTTCCTCCGCTTTAGCTGCTCCAGTCAACACT
 1027  ---+---------+---------+---------+---------+---------+------ 1086
       AGATAAAAATGACGACAAAATAAGCGACGAAGGAGGCGAAATCGACGAGGTCAGTTGTGA
       SerIlePheThrAlaValLeuPheAlaAlaSerSerAlaLeuAlaAlaProValAsnThr   24

       ACCACTGAAGATGAAACGGCTCAAATTCCAGCTGAAGCTGTCATCGGTTACTCTGATTTA
 1087  ---+---------+---------+---------+---------+---------+------ 1146
       TGGTGACTTCTACTTTGCCGAGTTTAAGGTCGACTTCGACAGTAGCCAATGAGACTAAAT
       ThrThrGluAspGluThrAlaGlnIleProAlaGluAlaValIleGlyTyrSerAspLeu   44

       GAAGGTGATTTCGATGTTGCTGTTTTGCCATTTTCCAACTCCACCAATAACGGTTTATTG
 1147  ---+---------+---------+---------+---------+---------+------ 1206
       CTTCCACTAAAGCTACAACGACAAAACGGTAAAAGGTTGAGGTGGTTATTGCCAAATAAC
       GluGlyAspPheAspValAlaValLeuProPheSerAsnSerThrAsnAsnGlyLeuLeu   64

       TTTATCAATACTACTATTGCCTCCATTGCTGCTAAAGAAGAAGGTGTTTCTTTGGATAAA
 1207  ---+---------+---------+---------+---------+---------+------ 1266
       AAATAGTTATGATGATAACGGAGGTAACGACGATTTCTTCTTCCACAAAGAAACCTATTT
       PheIleAsnThrThrIleAlaSerIleAlaAlaLysGluGluGlyValSerLeuAspLys   84
                                                 3'-CCACAAAGAAACCTATTT

                                                HindIII
                                      5'-TTGGTTGAAGCTTTGTACTTGGTTTGC
       AGATTCGTTAACCAACACTTGTGCGGTTCCCACTTGGTTGAAGCTTTGTACTTGGTTTGC
 1267  ---+---------+---------+---------+---------+---------+------ 1326
       TCTAAGCAATTGGTTGTGAACACGCCAAGGGTGAACCAACTTCGAAACATGAACCAAACG
       ArgPheValAsnGlnHisLeuCysGlySerHisLeuValGluAlaLeuTyrLeuValCys  104
       TCT  GCAATTGGTTGTGAACACGCCAAGGGTGAACCAACTTCGAAACATGAACC-5'
         C  A                  #593 ↑
        T   ATGTAGCCTTTGGT
       T                  T          TGACGATGCT
       CTTCGACTTCGACTTCGAC           C        A
                          #3075 ↓    T        G
       GGTGAAAGAGGTTTCTTCTACACTCCTAAG         AGGTATTG-3'
       GGTGAAAGAGGTTTCTTCTACACTCCTAAGGCTGCTAAGGGTATTGTCGAACAATGCTGT
 1327  ---+---------+---------+---------+---------+---------+------ 1386
       CCACTTTCTCCAAAGAAGATGTGAGGATTCCGACGATTCCCATAACAGCTTGTTACGACA
       GlyGluArgGlyPhePheTyrThrProLysAlaAlaLysGlyIleValGluGlnCysCys  124

       ACCTCCATCTGCTCCTTGTACCAATTGGAAAACTACTGCAACTAGACGCAGCCCGCAGGC
 1387  ---+---------+---------+---------+---------+---------+------ 1446
       TGGAGGTAGACGAGGAACATGGTTAACCTTTTGATGACGTTGATCTGCGTCGGGCGTCCG
       ThrSerIleCysSerLeuTyrGlnLeuGluAsnTyrCysAsn***     3'-GGGCGTCCG  138
```

Fig. 2a

```
  XbaI
  TCTAGAAACTAAGATTAATATAATTATATAAAAATATTATCTTCTTTTCTTTAT-3'
1447 ---+---------+---------+---------+---------+---------+    1500
  AGATCTTTGATTCTAATTATATTAATATATTTTTATAATAGAAGAAAAGAAATA-5'
  AGATCTTTGATTCTAATT-5'
       #2371 ↑
```

Fig. 2b

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 96/00107

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C07K 14/62, A61K 28/28
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

REG, CAPLUS, MEDLINE, WPI

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 9507931 A1 (NOVO NORDISK A/S), 23 March 1995 (23.03.95) --	1-11
X	Patent Abstracts of Japan, Vol 14,No 7, C-673, abstract of JP, A, 1-254699 (KODAMA K.K.), 11 October 1989 (11.10.89) --	1-11
A	WO 9200321 A1 (NOVO NORDISK A/S), 9 January 1992 (09.01.92) --	1-24
A	EP 0376156 A2 (HOECHST AKTIENGESELLSCHAFT), 4 July 1990 (04.07.90) -- --------	1-24

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 July 1996	15 -07- 1996
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Carolina Gömez Lagerlöf Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 96/00107

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 27-28
because they relate to subject matter not required to be searched by this Authority, namely:

See PCT Rule 39.1(iv): Methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

01/04/96

International application No.

PCT/DK 96/00107

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A1- 9507931	23/03/95	NONE	
WO-A1- 9200321	09/01/92	AU-A- 8054391	23/01/92
		EP-A,A- 0536245	14/04/93
		JP-T- 5508406	25/11/93
EP-A2- 0376156	04/07/90	AT-T- 136038	15/04/96
		AU-B,B- 623963	28/05/92
		AU-A- 4733289	05/07/90
		CA-A- 2006818	29/06/90
		DE-A- 3844211	05/07/90
		DE-D- 58909633	00/00/00
		IL-A- 92905	21/10/94
		JP-A- 2225498	07/09/90
		PT-B- 92757	29/12/95
		US-A- 5506202	09/04/96